The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) Prescreen Evaluation on Five *In Vitro* Pyrogenicity Assays (PBMC/IL-6; WB/IL-1; cryo WB/IL-1; WB/IL-6; MM6/IL-6) Submitted for Evaluation to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) by the European Centre for the Validation of Alternative Methods (ECVAM)

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SUMMARY

In June 2005, Background Review Documents (BRDs) detailing five *in vitro* human blood cell pyrogenicity tests were submitted by the European Centre for the Validation of Alternative Methods (ECVAM) as replacement tests for the currently required tests (i.e., rabbit pyrogen test and the bacterial endotoxin test; BET). These test methods are similar to each other in that they involve the measurement of cytokine levels from human blood cells or a human monocytoid cell line. The validation database for each test method consisted of the same 13 pyrogen-free, marketed, parenteral pharmaceuticals (10 for accuracy evaluations and 3 for reliability evaluations), each spiked with multiple concentrations of a bacterial endotoxin standard. Accuracy was determined by comparison of the results generated using a prediction model to the "true status" of the samples. The adequacy of each submission was evaluated based on 1) the extent to which the submissions provide the information requested in the ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods (NIH Pub. No. 03-4508); and 2) the extent to which the submissions address the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) prioritization criteria. With the exception of specific monetary cost, the BRDs addressed the ICCVAM prioritization criteria, and it appears that there are sufficient data to warrant an independent evaluation of the relevance and reliability of each of the five *in vitro* pyrogenicity test methods. However, minor deficiencies in the organization and content of the BRDs and supporting information were noted that should be corrected prior to a formal review by an expert peer review panel.

1.0 INTRODUCTION

In June 2005, the European Centre for the Validation of Alternative Methods (ECVAM) submitted five *in vitro* human blood cell pyrogenicity tests to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) for consideration by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as replacement tests for the currently required *in vivo* rabbit test or an *in vitro* test that requires the use of horseshoe crabs (bacterial endotoxin test; BET). A list of key references is included in **Section 3.0**.

Although the same cells are used for more than one test method, each procedure is considered to be a separate test method. However, these test methods are similar in that each assay involves the measurement of cytokine levels from either human blood, peripheral blood mononuclear cells (PBMC), or a human monocytoid cell line, as a biomarker of a pyrogenic response. In each assay, cytokine levels are measured with an enzyme-linked immunosorbent assay (ELISA). The five *in vitro* pyrogenicity test methods are identified as follows:

- PBMC/IL-6 (The Human PBMC/IL-6 *In Vitro* Pyrogen Test)
- WB/IL-1 (The Human Whole Blood/IL-1 *In Vitro* Pyrogen Test)
- cryo WB/IL-1 (The Human Whole Blood/IL-1 *In Vitro* Pyrogen Test: Application of cryopreserved human whole blood)
- WB/IL-6 (The Human Whole Blood/IL-6 *In Vitro* Pyrogen Test)
- MM6/IL6 (An Alternative *In Vitro* Pyrogen Test Using the Human Monocytoid Cell Line MONO MAC-6 [MM6])

1.1 Test Method Validation Database

A total of 13 test substances were used in the validation study made up of currently marketed parenteral drugs that have been determined to contain no detectable pyrogens. The positive control was the 2nd International World Health Organization (WHO) Standard for endotoxin (i.e., from *Escherichia coli* 0113:H10:K- [94/580]), 0.5 endotoxin

units [EU]/mL in clinical stock saline solution, while the negative control was 0.9% clinical stock saline solution. For the accuracy evaluation, 10 test substances (**Table 1**) were spiked with five spike solutions (0, 0.25, 0.5, 0.5, and 1.0 EU/mL) and tested once in three different laboratories. The spike solutions were made with the same endotoxin standard used in the positive control. Accuracy was determined by comparison of the results generated using the prediction model to the "true status" of the samples. The absorbance of each "unknown" sample was compared to that of an endotoxin standard curve. The samples were classified as either negative or positive based on the assigned pyrogen threshold value (0.5 EU/mL). For the reliability analysis, 3 test substances (**Table 2**) were spiked with four different spike solutions (0, 0, 0.5, 1.0 EU/mL) and tested 3 times in 3 different laboratories.

Table 1[1]. Substances used for evaluating test method accuracy.

Drug Name	Source	Agent	Indication
Glucose 5% (w/v)	Eifel	Glucose	Nutrition
Ethanol 13% (w/w)	B. Braun	Ethanol	Diluent
MCP®	Hexal	Metoclopramid	Antiemetic
Orasthin®	Aventis	Oxytocin	Initiation of delivery
Binotal®	Aventis	Ampicillin	Antibiotic
Fenistril®	Novartis	Dimetindenmaleat	Anti-allergy
Sostril®	GlaxoSmithKline	Ranitidine	Anti-acidic
Beloc®	Astra Zeneca	Metoprolol tartrate	Heart dysfunction
Drug A[2]	-	0.9% NaCl	-
Drug B[2]	-	0.9% NaCl	-

[1]Table 1 modified from Table 3.3.1 of each BRD.
[2]The BRDs indicate that Drugs A and B were included as saline controls using "notional ELCs"

Table 2[1]. Substances used for evaluating test method reliability.

Drug Name	Source	Agent	Indication
Gelafundin®	Braun melsungen	Gelatin	Transfusion
Jonosteril®	Fresenius	Electrolytes	Infusion
Haemate®	Aventis	Factor VIII	Hemophilia

[1]Table 2 modified from Table 3.3.2 of each BRD.

There are no direct comparisons of the proposed *in vitro* test methods to either the rabbit pyrogen test or the bacterial endotoxin test. Historical data from 171 rabbits tested with

endotoxin (0, 5, 10, 15, 20 EU/kg in 1 mL/kg) were obtained. The endotoxin was obtained from 2 sources: 1) *E. coli* EC5; 2) *E. coli* EC6 (reportedly identical to the WHO standard used in the validation studies). From these data, it was established that 50% of the rabbits got fever within 180 minutes of injection with 5 EU/kg. Based on the largest allowable volume for injection in rabbits (10 mL/kg), the limit of detection that alternative pyrogen tests must meet was defined as 0.5 EU/mL. A "theoretical" measure of performance of the rabbit pyrogen test was established for comparison to the *in vitro* test methods. Taking into account the prevalence of the 5 spike solutions and calculating the probabilities of misclassification using the defined threshold of pyrogenicity (i.e., 0.5 EU/mL), the theoretical sensitivity was calculated as 75%, and the theoretical specificity was 96%.

2.0 NICEATM PRESCREEN EVALUATION OF THE FIVE *IN VITRO* PYROGENICITY TEST METHODS

A Background Review Document (BRD) was submitted for each *in vitro* pyrogenicity test method. The five individual BRDs were reviewed for completeness and to identify aspects or omissions that could impede an expert peer review. The BRDs were not reviewed with respect to data quality or presentation, or validation study conclusions. Rather, the adequacy of each submission was evaluated based on the following criteria:

1) The extent to which the submissions provide the information requested in the ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods (NIH Pub. No. 03-4508).
2) The extent to which the submissions address the following ICCVAM prioritization criteria:
    - The extent to which the proposed test methods are:
        - Applicable to regulatory testing needs
        - Applicable to multiple agencies/programs
        - Warranted, based on the extent of expected use or application and impact on human, animal, or ecological health
    - The potential for the proposed test methods, compared to current test methods

accepted by regulatory agencies, to:

- Refine animal use (decrease or eliminate pain and distress)
- Reduce animal use
- Replace animal use

- The potential for the proposed test methods to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies
- The extent to which the test methods provide other advantages (e.g., reduced cost and time to perform) compared to current methods

Due to the similarities among the five test methods, much of the information contained in each BRD relevant to the ICCVAM prioritization criteria is duplicative. For this reason, unless otherwise indicated, the responses included below are relevant to all five test methods.

2.1 Applicability to Current U.S. and European Union (EU) Regulatory Testing Needs

There are current regulatory requirements to test pharmaceuticals and other products (e.g., medical devices) for pyrogenicity (**Tables 3** and **4**). The pyrogenicity assays that are currently acceptable to regulatory authorities require intact animals (rabbits) or an *in vitro* test that requires the use of horseshoe crabs (BET). According to the BRDs, "dependent on the product and the presence of relevant clinical data on unexpected pyrogenicity of clinical lots, the proposed test method[s] may be an alternative method for pyrogen testing, thus substituting [for] the rabbit pyrogen test or the BET. In certain cases, the proposed test method may function as a supplementary test method to assess compliance to the licensing dossier. In case the proposed test method [s] is an alternative for pyrogenicity testing, a thorough cross-validation between the proposed test method and the original method for the specific medicinal product is warranted. In case the proposed test method[s] is an adjunctive test to screen for (unexpected) pyrogenic lots,

159 **Table 3. Regulations/Guidance Documents on the Requirements for Pyrogenicity**
160 **Testing**
161

Regulation/Guideline	Pyrogenicity Testing Requirements
21CFR610.13 – Purity. (April 1, 2005)	"Each lot of final containers of any product intended for use by injection shall be tested for pyrogenic substances by intravenous injection into rabbits as provided in paragraphs (b) (1) and (2) of this section: *Provided*, That notwithstanding any other provision of Subchapter F of this chapter, the test for pyrogenic substances is not required for the following products: Products containing formed blood elements; Cryoprecipitate; Plasma; Source Plasma; Normal Horse Serum; bacterial, viral, and rickettsial vaccines and antigens; toxoids; toxins; allergenic extracts; venoms; diagnostic substances and trivalent organic arsenicals."
FDA - Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (December, 1987)	"This guideline sets forth acceptable conditions for use of the Limulus Amebocyte Lysate test. It also describes procedures for using this methodology as an end-product endotoxin test for human injectable drugs (including biological products), animal injectable drugs, and medical devices. The procedures may be used in lieu of the rabbit pyrogen test." "On the basis of extensive experience in review of LAL data on devices since November 1977, CDRH believes that the LAL test, when validated according to this guideline, is at least equivalent to the rabbit pyrogen test as an end-product test for medical devices. A manufacturer labeling a device as non-pyrogenic must validate the LAL test for that device in the test laboratory to be used for end-product testing before using the LAL test as an end-product endotoxin test for any device." IV. Human and Animal Drugs and Biological Products "A batch which fails a validated LAL release test should not be retested by the rabbit test and released if it passes. Due to the high variability and lack of reproducibility of the rabbit test as an endotoxin assay procedure, we do not consider it an appropriate retest procedure for LAL failures." V. Medical Devices "Manufacturers may retest LAL test failures with the LAL test or a USP rabbit pyrogen test. If the endotoxin level in a device eluate has been quantitated by LAL at 0.5 EU/mL endotoxin or greater, then retest in rabbits is not appropriate."
FDA - Guidance for Reviewers: Instructions and Template for Chemistry, Manufacturing, and Control (CMC) Reviewers of Human Somatic Cell Therapy Investigational New Drug Applications (INDs) (August 2003)	"Endotoxin testing using the Limulus Amebocyte Lysate (LAL) assay method is typically done as an alternative to pyrogenicity testing (see 21 CFR 610.13(b)) for early-phase trials. If the sponsor is using the LAL endotoxin method, you should inform the sponsor that, for licensure, the LAL endotoxin test must be shown, as explained in 21 CFR 610.9, to be equivalent to that of the pyrogenicity test described in 21 CFR 610.13(b)."
FDA - Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications (February 2005)	"We recommend that you perform a test for pyrogenic substances and that you include the test results with the bulk release documentation. The Limulus Amebocyte Lysate (LAL) test is a sensitive indicator of the presence of bacterial endotoxins and endotoxin contamination should not exceed 5.0 EU/kg body weight for the intended recipients."

Regulation/Guideline	Pyrogenicity Testing Requirements
FDA - Guidance for FDA Review Staff and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) (November 2004)	"Endotoxin testing using the Limulus Amebocyte Lysate (LAL) assay method is typically done to detect pyrogens (endotoxin) for products in early-phase clinical trials, and for marketed products. If you are using the LAL endotoxin method, the process for manufacture may also need to be evaluated for production of intrinsic pyrogenic substances other than endotoxin using the pyrogenicity test described in 21 CFR 610.13 (b)."
FDA - Guidance for Industry and/or for FDA Reviewers/Staff and/or Compliance: Preparation of a Premarket Notification Application for a Surgical Mesh (March 1999)	In accordance with the Blue Book Guidance G95-1 ("Use of International Standard ISO-10993, 'Biological Evaluation of Medical Devices Part 1: Evaluation and Testing'"), acceptable test results should be supplied for … pyrogenicity. If the [device] is to be labeled "pyrogen free" or "nonpyrogenic," satisfactory results from the USP pyrogen test (rabbit) or an equivalent test, performed on the final end product, should be provided and lot release criterion for pyrogenicity need to be identified.
FDA- Center for Veterinary Medicine Program Policy and Procedures Manual (Guide 1240.4122, 4/25/00)	"The United States Pharmacopeia (USP) has recognized the Limulus Amebocyte Lysate (LAL) method as the official method for assaying drug products for lipopolysaccharides produced by gram negative microorganisms (bacterial endotoxins). The rabbit pyrogen test may be used only if a product is incompatible with the LAL test. The CVM endorses this position. However, during the development of a product and the manufacturing process validation (the first 3 commercial batches manufactured), the product should be assayed by both the LAL test and the rabbit pyrogen test. This is because there is the possibility of the presence of pyrogenic materials in the product that are not lipopolysaccharides. Testing the first 3 commercial batches would demonstrate if pyrogen contamination other than lipopolysaccharides is present in the final drug product. After the first 3 commercial lots, provided the rabbit pyrogen testing is negative, the LAL test should be utilized for release testing."
USP XXII ⟨1041⟩ Biologics (1990)	"No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (see *Safety Tests-General* under *Biological Reactivity Tests*, *In vivo* ⟨88⟩, *Sterility Tests* ⟨71⟩, *Water Determination* ⟨921⟩, and *Pyrogen Test* ⟨151⟩, as well as *Bacterial Endotoxins Test* ⟨85⟩)."

Table 4. Personal Communications Regarding Regulatory Testing Requirements for Pyrogenicity

Agency/Center	Pyrogenicity Testing Requirements
FDA-CBER	The FDA acknowledges that the rabbit pyrogenicity test and Limulus bacterial endotoxin test (BET) do not measure the same thing. The BET is a test for endotoxin where as the rabbit test will detect any contaminant in a product that is pyrogenic. Generally the BET assay for endotoxin is adequate, but it really depends on the manufacturing process. If the FDA believes that the process introduces impurities/contaminates that have the potential to be pyrogenic or we are uncertain as to whether this will be the case we can then ask the sponsor to do testing according to CFR 610.13 for licensure. During product development (early phase IND) sponsors are asked to test for endotoxin, for which the BET is recommended
FDA-CDER	While the BET is currently accepted, it is not a full replacement for the *in vivo* rabbit pyrogen test. Rather it is used/accepted whenever considered appropriate. Although it is highly sensitive, the failure of the BET to detect non-endotoxin pyrogens as well as its susceptibility to interference (e.g., high protein levels of test substances) prevents it from being considered a full replacement.
FDA-CDRH	CDRH requires the rabbit test for all new materials before the device is cleared for marketing. However, once the device is approved, the BET can be used as routine test for the presence of endotoxins, which is required for all implants and devices contacting the blood and CSF.
European Commission – Joint Research Centre	There are still circumstances under which the rabbit pyrogenicity test would still be required (e.g., a product that interferes with the BET; when non-endotoxin pyrogens might contaminant the product). Examples of products that currently require the rabbit pyrogen test in the EU include parenteral preparations, Haemophilus B vaccine, Hepatitis B vaccine, Pneumococcal vaccines, rabies for human use, tick-borne encephalitis, human immunoglobulins, human albumin, blood products as coagulation factor VII, VIII, IX, XI, human plasma, and prothrombin.

alert and alarm limits may be established based on consistency of production lots or (preferably) based on actual clinical data."

2.2 Applicability to Multiple Agencies or Programs

These methods will reportedly be applicable to all agencies and programs that require pyrogenicity testing of pharmaceuticals and other products. The U.S. Food and Drug Administration (FDA) Center for Biologic Evaluation and Research (CBER), Center for Drug Evaluation and Research (CDER), Center for Devices and Radiological Health (CDRH), and Center for Veterinary Medicine (CVM) require that human injectable drugs (including biological products), animal injectable drugs, and medical devices be tested for the presence of pyrogenic substances.

2.3 Extent of Expected Use or Application and Impact on Human Health

As detailed in **Section 2.1**, under certain circumstances the proposed tests are intended to replace tests that are used extensively in pharmaceutical development (i.e., *in vivo* rabbit pyrogen test, BET). They are allegedly as good as, if not better than, current test methods for identifying both endotoxin and non-endotoxin pyrogens (see **Section 2.5**). Therefore, they may offer improved prediction of pyrogenicity and subsequently provide greater protection of human health.

2.4 The Potential for the Proposed Test Method, Compared to Current Accepted Test Methods, to Refine, Reduce, or Replace Animal Use

As stated in **Section 2.3**, the proposed test methods are intended to replace tests that are used extensively in pharmaceutical development. The two most common pyrogen tests presently used (i.e., *in vivo* rabbit pyrogen test, BET) require the use of animals. While the BET is most often performed using blood drawn from *Limulus polyphemus* (the horseshoe crab) which are subsequently returned to the wild, a portion of these animals do not survive the procedure (which requires approximately 20% of the total blood volume, according to the BRD). The proposed test methods will reduce and replace animal use because they rely on human blood cells or a human monocytoid cell line that can be isolated and cultured in the test laboratory.

2.5 The Potential for the Proposed Test Method to Provide Improved Prediction of Adverse Health Effects, Compared to Current Accepted Test Methods

Sufficient data are presented to allow an assessment of the predictivity of the proposed test methods. Because these test methods are conducted using cells of human origin, the submitter contends that they will better reflect the human physiological response than current methods (i.e., *in vivo* rabbit pyrogen test, BET), and thus more effectively predict adverse effects in humans. It is not clear if they would also provide improved predictivity of adverse effects in animals (i.e., when testing veterinary pharmaceuticals).

2.6 The Extent to Which the Test Method Provides Advantages (e.g., Reduced Cost and Time to Perform) Compared to Current Methods

Specific cost requirements are not provided, and therefore a determination of relative costs cannot be made. The BRD cites two factors in contributing to the cost of the proposed test methods: reagent costs and labor costs. Because the proposed test methods are reportedly more labor-intensive than current methods (i.e., *in vivo* rabbit pyrogen test, BET), the costs are anticipated to be greater. However, the proposed methods do appear to be adaptable to higher throughput, which could make them more cost effective.

The proposed test methods are estimated to require approximately two working days. On day one, test materials are prepared and incubated with the relevant blood cells/cell line. The immunoassay for the appropriate cytokine is conducted on day 2. In comparison, both the BET and the rabbit pyrogen test can be completed in one day. However, prior to a rabbit's first use in a pyrogen test, a sham test (i.e., includes all steps but the injection) must be performed. In addition, positive results in the first three rabbits tested are to be followed by testing in an additional five animals. Such circumstances could cause testing to extend into a second workday.

2.7 Conclusion

With the exception of specific monetary cost, the BRDs addressed the ICCVAM prioritization criteria, and it appears that there are sufficient data to warrant an independent evaluation of the relevance and reliability of each of the five *in vitro* pyrogenicity test methods. However, minor deficiencies in the organization and content of the BRDs and supporting information were noted that should be corrected prior to a formal review by an expert peer review panel.

3.0 Key References

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